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(54) Title: NERVE GROWTH PEPTIDES AND USES THEREFOR (57) Abstract This invention describes novel molecules capable of promoting nerve growth, and therapeutic methods for promoting nerve growth using these molecules. Molecules of this invention include a protein fragment substantially homologous to an active region of the brain glycoprotein ependymin. The active region is primarily responsible to the nerve growth promoting capacity of these molecules. The molecules of this invention may be administered to a subject to correct genetic and systemic disorders and to treat nervous systems disorders resulting from trauma.		

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-1-

NERVE GROWTH PEPTIDES AND USES THEREFORTechnical Field

This invention is in the field of neurobiology and neurochemistry.

Background

The survival, growth, maintenance and function of certain classes of nerve cells during development are believed to depend on nerve growth factors. A molecule that functions as a nerve growth factor is ependymin.

Ependymin is a glycoprotein that is synthesized in the brain by specific cells which secrete and maintain a steady-state concentration of the protein in the brain extracellular (ECF) and cerebrospinal (CSF) fluids. Ependymin comprises dimers of two polypeptide chains (alpha, M.W. 37,000 and beta, M.W. 31,000) linked by disulfide bonds. These are converted to the gamma-chain (M.W. 26,000) in ECF. The amino acid sequences of the alpha, beta and gamma chains are substantially identical. Ependymins are initially released into the extracellular spaces of the brain as mixtures of $\alpha\beta$ dimers (M.W. 68,000) which become converted to $\beta\gamma$ (M.W. 58,000) and γ_2 (M.W. 53,000) dimers.

SUBSTITUTE SHEET

-2-

Shashoua, V.E., J.Physiol. (Paris) 83:232-329 (1989). The mode by which ependymin functions as a nerve growth promoter is not known. Ependymin has been implicated in the consolidation process of long-term memory formation. V.E. Shashoua, Cell. Molec. Neurobiol., 5: 183-207 (1985).

For nerve growth factors to be therapeutically useful, they must be delivered to an active site in the body. This requirement is beset with several difficulties. First, although many nerve growth factors are peptides, P.L. McGeer et al., "Molecular Neurobiology of the Mammalian Brain", 2nd Edition, Plenum Press, 1987, the particular amino acid sequences responsible for their nerve growth-promoting properties are either unknown or are poorly characterized.

Second, ingestion of a drug containing a nerve growth factor often is not possible because many drugs will not survive the environment of the stomach. Thus, easy and safe self-administration of many drugs is not available. A drug, of course, can be injected directly into the bloodstream of the patient. However, because some drugs do not survive for very long in the bloodstream, frequent injections may be necessary, at great inconvenience to the patient. The inability of a drug to survive in the bloodstream can also be overcome in certain

SUBSTITUTE SHEET

-3-

instances by increasing the dosage. Unfortunately, increasing the dosage can result in undesirable side effects.

The delivery of a nerve growth factor such as ependymin into the central nervous system (CNS) via the bloodstream involves an additional and extraordinary obstacle; the nerve growth factor must be capable of crossing the blood-brain barrier. The blood-brain barrier may be regarded as a biological exclusion barrier involving both passive and active transport, the barrier controlling the exchange of materials between the plasma and the central nervous system. Many drug substances, including ependymin, are unable to pass through this barrier in efficacious amounts, or at all. Therefore, attempts to increase the level of ependymin in the central nervous system by introducing it into the bloodstream are not successful because it does not cross the blood-brain barrier. Therefore, it would be useful to develop molecules having the same, or greater, activity as ependymin that could be adapted easily to be introduced into the central nervous system.

Summary of the Invention

The invention provides novel molecules that are protein fragments capable of stimulating nerve growth. The protein fragments correspond to amino

-4-

acid sequences of naturally-occurring proteins and are no greater than about 80 amino acids in length. Proteins from which the nerve growth stimulating fragments are derived include neuronotrophic proteins such as epidermal growth factor, tubulin, nerve growth factor (NGF), laminin, fibronectin, NCAM and ependymin.

The protein fragments are preferably about 16 amino acids in length and contain sequences substantially homologous with one or more active regions of ependymin. Active regions of ependymin include amino acid sequences of from 5 to about 8 amino acid residues. These sequences of the ependymin active regions are located within two, non-contiguous neuroactive domains of ependymin. Active regions are associated with the N-terminal domain of the ependymin chain, particularly the first eighty amino acid residues. Active regions are also associated with the C-terminal domain of ependymin, in particular between amino acid residues 130 and 195. Preferably, the protein fragments contain sequences that are between about 70% and 100% homologous with the respective active region sequences of ependymin.

A preferred embodiment of the invention is an ependymin fragment containing amino acids from an active region of an ependymin neuroactive domain,

-5-

the fragment having a sequence substantially homologous to at least one protein fragment other than ependymin. The protein fragment sequences substantially homologous with ependymin active region sequences are primarily responsible for the nerve growth stimulating properties of the protein fragments.

The protein fragments of the invention can also be coupled to one or more secondary molecules, which molecule can include a second protein fragment derived from the original protein and/or from another protein. The ependymin fragment of the preferred embodiment can be coupled to one or more secondary molecules which can be either amino acid sequences from another ependymin fragment, sequences from a separate protein, or a non-proteinaceous moiety.

A preferred secondary molecule of the present invention is a pharmaceutically acceptable carrier whose linkage to the protein fragment is not degraded in the environment of the stomach and the bloodstream and, therefore, can be administered orally. The carrier also passes readily through the blood-brain barrier delivering the protein fragments of this invention into the central nervous system. The combination of protein fragment and carrier is called a prodrug. Prodrugs are formed by chemically

SUBSTITUTE SHEET

-6-

linking a suitable non-proteinaceous carrier to the protein fragments of the invention, the carrier being selected such that it has a brain penetration index of at least two times the brain penetration index of the protein fragment alone. Upon entering the central nervous system, prodrug can remain intact or the chemical linkage between the carrier and the protein fragment may be hydrolyzed, thereby separating the carrier from the fragment to release the nerve growth-stimulating fragment. The carrier preferably is a normal component of the central nervous system and is inactive and harmless. The preferred carriers are fatty acids, cholesterol, and glucose derivatives.

The present invention also discloses therapeutic methods for promoting nerve growth using the protein fragments of this invention. The methods include administering to a patient a protein fragment having a sequence that is substantially homologous to an active region of a neuroactive domain of ependymin. An embodiment of the method of the present invention uses sustained release delivery systems to administer the protein fragment(s) into the blood stream or a target tissue.

Through use of the compositions and methods of the present invention, nerve growth can be modulated to correct genetic and systemic disorders and to

-7-

treat nervous system disorders resulting from trauma.

Detailed Description of the Drawings

Figure 1 is a graph showing the dose-response effect of the intact ependymin molecule on neurite growth of neuroblastoma cells.

Figure 2 is a graph showing the dose-response effect of a peptide fragment comprising the sequence I.D. No. 27 on neurite growth of neuroblastoma cells.

Figure 3 is a graph showing the effect of a protein fragment including sequence I.D. No. 26, and its mono-, and di-docosohexa-enoic acid derivatives on neurite growth of neuroblastoma cells.

Figure 3A shows the effect of sequence I.D. No. 27 on neurite growth.

Figure 3B shows the effect of the mono-docosohexa-enoic acid derivative of sequence I.D. No. 27 on neurite growth.

Figure 3C shows the effect of the di-docosohexa-enoic acid derivative of sequence I.D. No. 27 (R89-131) on neurite growth.

Detailed Description of the Invention

The present invention provides protein fragments capable of promoting nerve growth. The term "promoting nerve growth" means the ability of the protein fragment to stimulate nerve sprouting,

-8-

stimulate nerve elongation, and/or promote activities collateral thereto. Specifically, "collateral" activities are meant to encompass extracellular matrix formation. Extracellular matrix formation can define the locus at which changes in existing synaptic elements or growth of new synapses will take place. This extracellular matrix may provide a pathway for development of new neuronal connections. See generally, V.E. Shashoua, "The Role of Brain Extracellular Proteins in Neuroplasticity and Learning", Cell. Mol. Neurobiol. 5: 183-207 (1985). See also P.L. McGeer *et al.*, supra, pp. 419-420, describing neural cell adhesive molecules, or NCAM's that are instrumental in attaching outgrowing nerve fibers to extracellular matrices that guide nerve fibers to their targets.

The protein fragments of the present invention are amino acid sequences of one or more portions of a protein. The term "protein fragment" means portions substantially less than the entire naturally-occurring protein. The protein fragments preferably are no larger than about 80 amino acids long, and most preferably are between about 5 and about 16 amino acids long. The term includes protein fragments of this length and functional equivalents thereof. The term "functional equivalents" is meant to encompass any modifications in peptide bonding,

-9-

deletions, and/or substitutions to the amino acid sequences, or any deletions, and/or substitutions to the nucleotide sequences that code for these amino acids, such that the protein fragment, although it may have different physical properties, is within the fragment length disclosed herein and still is capable of stimulating nerve growth.

Applicants have discovered that the protein fragments of the invention include selected amino acid sequences that are substantially homologous to one or more "active regions" of ependymin. The term "substantially homologous" is meant to include protein fragment sequences that have at least about 70% homology to a given active region. The term is therefore meant to include sequence homologies that range from about 70% homology to complete homology (100%).

These active regions have been associated with the nerve growth-promoting properties of ependymin. The protein fragments of the invention may consist of a larger peptide chain, a smaller part of which corresponds to an ependymin active region. Alternately, the fragment may correspond entirely to the active region. The "active region" is preferably a short sequence of ependymin of between about 5 and about 8 amino acids in length.

.....The protein fragments of the invention are

-10-

substantially homologous to active regions of ependymin that are located with one or more neuroactive domains of ependymin. One neuroactive domain contains ependymin active regions that have significant effects in promoting nerve elongation and nerve sprouting. Preferred active region sequences derived from this neuroactive domain of the ependymin molecule are associated with the N-terminal region of the ependymin chain, in particular between amino acid residues 1 and 80. Proteins containing fragments thereof that are substantially homologous to active regions in this ependymin neuroactive domain include proteins such as epidermal growth factor (EGF), EGF receptor, nerve growth factor (NGF), laminin, and integrin.

Another neuroactive domain of ependymin containing active regions substantially homologous to a protein fragment has effects on extracellular matrix formation. This domain is associated with the C-terminal end of the ependymin chain, preferably between amino acids residues 130 and 195. Proteins containing fragments that are substantially homologous to active regions in this ependymin neuroactive domain include laminin, NCAM, tubulin, and fibronectin.

The preferred active regions of the ependymin neuroactive domain effective in extracellular matrix

-11-

formation are shown in Table I along with the protein fragments to which these active regions are substantially homologous.

SUBSTITUTE SHEET

-12-

TABLE 1: Matrix-forming active regions

<u>I.D. Number</u>	<u>Ependymin active region^a</u>	<u>Protein fragment (homologous segment^b)</u>	<u>% Homology/Reference</u>
1	Asp Leu Gln Val Phe Val (165-170)	Human fibronectin (886-890)	83.3 (Kornbluth <i>et al.</i> <u>EMBO J.</u> , 4:1755 (1985)
2	Gly Glu Lys Lys Asp (146-151)	Fibronectin receptor (660-664)	100.0 (Argavos <i>et al.</i> , <u>J. Cell. Biol.</u> 105, (1987)
3	Ala Thr His Glu Ser (94-99)	Tubulin (yeast 2-3) (282-287)	100.0 (Schatz <i>et al.</i> , <u>Mol. Cell. Biol.</u> , 6:3711,
4	Cys Leu Pro ____ Val Ser Gly (138-148)	Laminin Human B1 (1056-1062)	71.4 (Sashi <i>et al.</i> , <u>PNAS, USA</u> , 84:935)
5	Leu Pro Val Ser Gly Ser (139-143)	NCAM (197-200)	83.3 (Dickson <i>et al.</i> , <u>Cell.</u> , 50:1189;
6	Pro Cys His Ala Pro Pro (6-11)	Integrin (human) (21-26)	83.3 (Horwitz & Hynes <u>Cell.</u> , 46:271;

a. Numbers in parenthesis refer to the amino acid residue number of the gamma-chain of ependymin.

b. Numbers refer to the amino acid residue number of the protein, the complete sequence of which is cited in the reference.

-13-

Similarly, Table II presents the preferred active regions of the ependymin neuroactive domain effective in stimulating nerve growth and elongation. The protein fragments to which these active regions are substantially homologous are shown as well. The proteins referred to in Tables I and II are collectively referred to hereinafter as "neuronotrophic" proteins.

-14-

TABLE 2: Nerve growth active regions

<u>I.D. Number</u>	<u>Ependymin active region^a</u>	<u>Protein fragment (homologous segment^b)</u>	<u>% Homology/Reference</u>
7	Gly Gly His Asp Leu Glu Ser Gly (23-30)	Mouse Epidermal Growth Factor (586-596)	75 (Arundi <u>et al.</u> , <u>J. Biochem.</u> , 29:365)
8	Asp Asp Leu Gln ___ Val Phe (164-169)	Epidermal Growth Factor (169-175)	85.7 (Arundi <u>et al.</u> , <u>J. Biochem.</u> , 29:365)
9	Pro Leu Thr ___ Ser Gly (11-15)	Human Epidermal Growth Factor, Receptor (691-696)	83.3 (Gregory & Preston, <u>Int. J. Pept. Prot. Res.</u> , 9:107)
10	Leu Ile His Phe Glu Glu Gly Val (59-66)	Human Epidermal Growth Factor (691-696)	75.0 (Gregory & Preston, <u>Int. J. Pept. Prot. Res.</u> , 9:107)
11	Gly Glu Phe Ser ___ Tyr Asp Ser (30-36)	Nerve Growth Factor (10-18)	75.0 (Scott <u>et al.</u> ; <u>Nature</u> , 301:538)
12	His Ala ___ Pro Pro Leu Thr Ser (8-14)	Nerve Growth Factor, Precursor (34-43)	75 (Scott <u>et al.</u> ; <u>Nature</u> , 301:538)
13	Asp Leu Glu Ser Gly Glu Phe (26-32)	Laminin B1 (Human and Mouse (126-131)	71.4 (Rocco <u>et al.</u> , <u>EMBO J.</u> , 6:2343)

a. Numbers in parenthesis refer to the amino acid residue number of the gamma-chain of ependymin.

b. Numbers refer to the amino acid residue number of the protein, the complete sequence of which is cited in the reference.

-15-

The protein fragments of the invention can include fragments that are substantially homologous with functional equivalents of the active regions. As described above, the resulting protein fragments may have different physical properties but still are capable of promoting nerve growth as defined herein. For example, preferred protein fragments substantially homologous to the active region between amino acid 30 and amino acid 36 of the ependymin molecule (Table II: sequence I.D. No. 11) can include, but are not limited to, fragments homologous to amino acid sequences such as sequence I.D. numbers 14 and 15.

The protein fragments of the invention are characterized in having one or more structural forms. In one embodiment, the protein fragments of the invention consist of fragments entirely (100%) homologous to ependymin active regions. These fragments include, but are not limited to, fragments identical to the amino acid sequences of ependymin referred to in Tables I and II as well as any functional equivalents of these active regions.

In another embodiment, the fragments are coupled at either or both of their N-terminal or C-terminal ends to other molecules, referred to herein as secondary molecules. These secondary molecules can be portions from the same protein as the original

SUBSTITUTE SHEET

-16-

protein fragment, from a completely different protein, or from a non-proteinaceous moiety. The fragment can be coupled to one or more secondary molecules via a group capable of being attached, directly or indirectly, to a carboxyl group of the secondary molecule. The carboxyl group of a secondary molecule can form, for example, an ester or amide bond with the nerve growth-stimulating protein fragment. Other couplings such as phosphoramidate, sulfate, sulfonate, peptide, phosphate or urethane can be used to couple the secondary molecule to the protein fragment as will be recognized by one of ordinary skill in the art.

The secondary molecule can be a segment of the protein fragment contiguous with the original protein fragment. The term "contiguous" means that the secondary molecule is derived from an adjacent portion of the original protein fragment. Since the protein fragment is substantially homologous to an ependymin active region within an ependymin neuroactive domain, a protein fragment substantially homologous to an active region of one ependymin domain, for example in Table I, can be coupled to a secondary molecule taken from a contiguous portion of the same neuroactive domain.

In embodiments of this type, therefore, a protein fragment substantially homologous with a

-17-

first active region of ependymin is preferably linked at either or both of its N-terminal and C-terminal ends to a secondary molecule substantially homologous to that portion of ependymin that is adjacent to, or closely associated with, the first active region. Referring to Table II, for example, a protein fragment of this type can include a fragment substantially homologous with active region sequence ID number 11 (amino acid residue 30 to amino acid residue 36) peptide bonded to a protein fragment substantially homologous to the portion of ependymin that is on either side of the active region (e.g. amino acid residues 23 to 29 and/or amino acid residues 37 to 43). Examples of this embodiment are sequence I.D. numbers 19 through 25. In particular, sequence I.D. number 19 is amino acid residue 23 to 29 of ependymin, sequence I.D. number 20 is a functional equivalent thereof, and sequence I.D. numbers 21-25 are protein fragments in which these sequences are peptide bonded to their adjacent active region. Sequence I.D. number 26 is amino acid residue 80-85 of ependymin, described in Examples 3 and 4, below.

The secondary molecule can also be derived from a non-contiguous portion of the original fragment, i.e. a non-adjacent portion, so that the respective fragments are substantially homologous with active

-18-

regions from different neuroactive domains of ependymin. Referring to Tables I and II, for example, a protein fragment of this type can include a fragment substantially homologous with active region sequence ID number 11 (amino acid residue 30 to amino acid residue 36) peptide bonded at either or both of its N-terminal and C-terminal ends to a protein fragment substantially homologous to active region sequence number 1 (amino acid residue 164 to amino acid residue 169). In this embodiment, the secondary molecule is a protein fragment derived from a different neuronotrophic protein than the original protein fragment.

In further embodiments of the invention, protein fragments substantially homologous with active regions derived from a neuroactive domain of ependymin can be coupled to a secondary molecule that is derived from a completely different protein that may not possess nerve growth stimulating properties. For example, a protein fragment can be coupled to a secondary molecule that is capable of targeting a specific nerve cell or nerve-innervated tissue within the body. Secondary molecules of this type may include, for example, tissue-specific proteins (e.g. neuron-specific enolase; PCP 9.5: Wilkinson *et al.*, Science 246: 670 (1989)) or tissue or cell specific antibodies (e.g. neuron-specific monoclonal antibodies: Klose, M. and D. Bentley,

-19-

Science 245: 982 (1989)).

Molecules of this invention also include one or more protein fragments that can be coupled, or otherwise bound to, a secondary molecule that is a carrier capable of delivering the fragment across the blood-brain barrier and effectively delivering the protein fragment of this invention to the central nervous system. The combination of a secondary molecule capable of crossing the blood-brain barrier and protein fragment(s) of this invention is referred to as a "prodrug".

Preferred secondary molecules useful in forming prodrugs are non-proteinaceous and are disclosed in co-pending U.S. applications serial Nos. 160,667 and 315,134, the disclosures of which are incorporated herein by reference. Such secondary molecules may be linked to the protein fragment(s) by chemical linkages that are hydrolyzed by enzymes in the central nervous system. In this way, the prodrug can cross the blood-brain barrier and can liberate the protein fragment of this invention by, for example, hydrolysis of the linkage between the secondary molecule and protein fragment. The non-toxic secondary molecule is then free to remain intact or be further metabolized by the brain. Alternately, it may not be necessary to release the protein fragment from its secondary molecule in order to obtain the desired results. Under these

SUBSTITUTE SHEET

-20-

circumstances, the prodrug can cross the blood-brain barrier and remains intact.

The preferred carriers of the invention are capable of delivering the nerve-growth promoting protein fragment across the blood-brain barrier in an efficient manner. A brain penetration index (BPI) determination is a useful criterion for measuring the capacity of a molecule to efficiently cross the blood-brain barrier. Shashoua, V.E., Jacob, J.N., Ridge, R., Campbell, A. and Baldessarini, R.J., J. Med. Chem. 27, 659 (1984). The BPI is a measure of the uptake of a compound by the brain relative to its uptake by the liver. The liver is chosen as a reference since it is an organ which presents no barrier to diffusable molecules present in the blood. The amount of material in the liver will reflect the amount available rather than the initial dose injected. Preferred carriers of the invention have a BPI at least twice that of the nerve growth promoting polypeptides.

Determination of the BPI is made by measuring the quantity of the compound (e.g. secondary molecule or polypeptide) present in the brain and liver five minutes after a subcutaneous injection, the equation being:

$$\text{BPI} = [\text{brain}]/[\text{liver}] \times 100$$

For these measurements, C¹⁴-labelled peptides

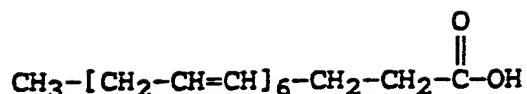
-21-

and secondary molecules can be synthesized (See e.g. Sigma® Radiochemicals Catalog - Sigma® Chemical Company, St. Louis, MO 63178), dissolved in 15% propylene glycol in 0.1 M NaHCO₃, and then injected subcutaneously (s.c.) into male balb C mice (20 ±2 g). After 5 minutes, the animals are sacrificed by cervical fracture and the brain and liver dissected out, weighed and homogenized in 8 and 10 ml, respectively, of Brain Protein Solvent (BPS) buffer [BPS = 2% sodium dodecyl sulfate in 0.03 M Tris, 6 M urea, (pH 7.6), 0.01 M EDTA and 0.14 N NaCl]. Aliquots can then be counted for ¹⁴C content in 10 ml of a liquid scintillation fluid (Liquiscent-National Diagnostic Company, Somerville, N.J.) using a Beckman liquid scintillation counter. The ¹⁴C counts are then used to calculate the total quantity of the compound present in the brain per gram of tissue as compared to that in the liver. The ratio of the amount in the brain as a percent of that present in liver is determined.

The preferred carrier can be a fatty acid which has between about 16 and about 26 carbon atoms, and more preferably between about 20 and about 24 carbon atoms. Most preferably, the secondary molecule is the all cis form of 4, 7, 10, 13, 16, 19 docosahexa-enoic acid:

SUBSTITUTE SHEET

-22-



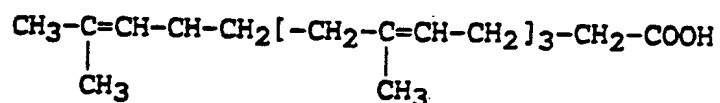
The length, the degree of saturation, and the extent to which the fatty acid is naturally occurring in the brain all affect the ability of the fatty acid to deliver the nerve-growth promoting polypeptide across the blood-brain barrier to an active site in the brain.

Fatty acids which are partially unsaturated and occur naturally in the brain are particularly preferred as secondary molecules (i.e. carriers). Fatty acids which occur naturally in the brain include those with 16 carbon atoms and 0, 1 or 2 double bonds (C16:0; C16:1; and C16:2), those with 18 carbon atoms and 1, 2 or 3 double bonds (C18:1; C18:2; and C18:3), those with 20 carbon atoms and 1, 2 or 4 double bonds (C20:1; C20:2; and C20:4) and those with 22 carbon atoms and 4, 5 or 6 double bonds (C22:4; C22:5; and C22:6). The position of the double bonds can be between any of the carbon atoms and the fatty acids, the preferred loci being those which occur naturally in the fatty acids of the central nervous system. The C16:0 and C22:6 are the preferred fatty acid carriers due to their preference for concentrating in the synaptosomal membranes, with C22:6 being most preferred. It also has been found that C18:3 acts above average in its

-23-

ability to deliver a compound across the blood-brain barrier.

Branched chain fatty acids having between 16 and 26 carbon atoms can also be used within the present invention. A hydrogen atom of the foregoing fatty acids can be replaced with a methyl, ethyl or isopropyl substituent at various positions along the carbon chain. Other, examples include the naturally occurring polyisoprenoids (dolichols) and analogs thereof such as



The protein fragments can be coupled to the fatty acid via a group capable of being attached, directly or indirectly, to the carboxyl group of the fatty acid. The carboxyl group of the fatty acid can form, for example, an ester or amide bond with the protein fragment. Other couplings such as phosphoramidate, sulfate, sulfonate, phosphate or urethane can be used to couple the fatty acid to the protein fragment as will be recognized by one of ordinary skill in the art.

Other secondary molecules besides fatty acids capable of introducing the protein fragments across the blood-brain barrier include inositol, 1-4

-24-

dihydropyridine, cholesterol, glucose and glucose derivatives. Preferred glucose derivatives are glucose esters such as 3-glucose succinoyl esters.

The preferred bonds for coupling secondary molecules to the protein fragments are those capable of surviving the environment of the stomach, thereby allowing oral administration of the prodrug. The preferred bonds also hydrolyze when the prodrug is introduced into the brain. An example of such a bond is believed to be an amide bond. The bonds incapable of surviving the environment of the stomach, e.g. ester bond, or also may be used to link the secondary molecule to the protein fragment. Prodrugs coupled with bonds such as these can be injected or protected from the environment of the stomach using coatings or liposomes, for example. Those skilled in the art would know how to coat such compounds for this purpose. Such a coating may be called for even in the presence of an amide bond between the carrier and the nerve growth factor.

The preferred method for facilitating bond coupling of the secondary molecules to the protein fragments of the invention is through one or more basic amino acids. These basic amino acid acids are preferably lysine and arginine. These amino acids can be part of the basic amino acid side chains of

SUBSTITUTE SHEET

-25-

the protein fragments of this invention shown, for example, in sequence I.D. numbers 2 and 17. Alternately, these basic amino acids are not part of the original protein fragment and can be peptide bonded to the N-terminal end of the fragment and/or the C-terminal terminal end of the fragment.

Various combinations of lysine and arginine can be employed to couple fragments of the invention with secondary molecules. Particularly preferred combinations of these basic amino acids include a pair of lysine residues bonded to the N-terminal end and/or the C-terminal end of the protein fragment; a single arginine residue bonded to the C-terminal end of the fragment; an arginine residue bonded to the N-terminal end of the fragment, which residue is bonded to a lysine; an arginine residue bonded to the C-terminal end of the fragment, which arginine residue is bonded to a lysine; and a single lysine residue bonded to the C-terminal end of the fragment.

An N-terminal lysine residue, in particular, has two amino groups with which a secondary molecule can form a bond. Thus, two terminal lysine residues that are peptide bonded to a fragment of the invention have a total of three amino groups with which a secondary molecule can form a bond: one N-terminal amino group of the fragment and two coupling sites available on the γ carbon of each

-26-

lysine residue. (See e.g. sequence I.D. No. 27). A di-substituted protein fragment (Lys-Lys) derivative can have a secondary molecule linked to either one of the three available amino groups, or it can be a mixture of all three forms with the average composition of a mono-substituent. A di-substituted protein fragment can be a mixture of all possible substitutions on 2 out of the 3 available sites. Because enzymes exist that hydrolyze the peptide bonds between the two lysines and the rest of the molecule, the growth-promoting protein may be liberated from the prodrug. In yet another embodiment of the invention, the secondary molecule may be coupled to the end of the peptide fragment through an amidated C-terminal carboxyl group ($-\text{CONH}_2$).

The protein fragment alone, or combined with a secondary molecule, is administered to a subject in a pharmaceutically acceptable amount. Alternately, a plurality of protein fragments, either alone or combined with secondary molecules, can be administered as a "cocktail" in a pharmaceutically acceptable amount. The term "subject" is intended to include mammals. The terms "pharmaceutically acceptable amount" is that amount which is capable of promoting nerve growth in the subject. A pharmaceutically acceptable amount can be determined on an individual basis and will be based, at least

SUBSTITUTE SHEET

-27-

in part, on consideration of the subject's size, severity of the symptoms to be treated, results sought, and specific secondary molecule used. Thus, a pharmaceutically acceptable amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

The molecules containing protein fragments of this invention may be used in vivo to treat peripheral or central nervous system nerve injury. By "nerve injury" is meant nerve damage caused by disease and/or physical or chemical trauma, including diseases such as Huntington's, Parkinson's, and Alzheimer's. The term is also meant to include other situations wherein nerve growth is to be facilitated. For example, the protein fragments may be useful in connection with the transplantation of healthy nerve tissue to a site of nerve injury. Pre-treatment of nerve tissue with molecules of this invention or transplantation in the presence of these molecules is likely to enhance nerve connectivity and growth.

Administration of the therapeutic molecules of this invention may be made by any method which allows the protein fragment to reach the target site of nerve injury. Typical methods include oral, rectal, peritoneal, subcutaneous, intravenous and

SUBSTITUTE SHEET

-28-

topical administration of the compounds. When the compounds are administered orally, the composition can be in the form of dragees, tablets, syrups or ampules. When the compounds are administered rectally, the composition can be in the form of a suppository. When the compounds of the invention are to be administered by topical application, they can be in the form of a gel. Physiologically acceptable vehicles for the therapeutic compositions of this invention include water, or aqueous solutions of non-toxic salts, such as saline or phosphate-buffered saline. Adjuvants, such as aluminum hydroxide gel, can also be employed. Also suitable are oil-in-water emulsions and compositions including organic solvents, such as dimethylsulfoxide (DMSO), propylene glycol, and glycerol. Solid materials can include nutritive materials such as sucrose or gelatin.

Other delivery systems can include sustained release delivery systems. Preferred sustained release delivery systems are those which can provide for release of the protein fragments of the invention in sustained release pellets or capsules. Many types of sustained release delivery systems are available. These include, but are not limited to: (a) erosional systems in which the protein fragment is contained in a form within a matrix, found in

SUBSTITUTE SHEET

-29-

U.S. Patent Nos. 4,452,775 (Kent) and 4,667,014 (Nestor et al.), the entire disclosures of which are incorporated herein by reference; and (b) diffusional systems in which an active component permeates at a controlled rate through a polymer, found in U.S. Patent Nos. 3,832,252 (Higuchi et al.) and 3,854,480 (Zaffaroni), the entire disclosures of which are incorporated herein by reference.

Methods of preparing the protein fragments of this invention include isolating the alpha, beta and gamma forms of ependymin from brain tissue and preparing an enzyme digest thereof. See V. E. Shashoua, Neurochemical Research, 13: 649-655 (1988). Individual peptide chains can be separated chromatographically. Shashoua, V.E. Brain Res. 122: 113-124 (1977).

Detailed sequence analysis of whole ependymin has been performed (Konigstorfer et al., J. Neurochem., 52:310-312 (1989). Sequence analysis on selected proteolytic digests of the ependymin chains can also be performed using routine methods. Briefly, alpha, beta or gamma-ependymin is subject to proteolysis and the digest is separated chromatographically. Selected digests of these chains, including the neuroactive domains are sequenced using routine methods. The entire amino acid sequences of many naturally occurring

SUBSTITUTE SHEET

-30-

neuronotrophic proteins are by now well known (see references in Tables I and II, the citations of which are incorporated herein by reference). The sequences derived from ependymin digests can be compared with known sequences from a wide variety of neuronotrophic proteins using computer database analysis.

Protein fragments and functional equivalents thereof that are identified as being substantially homologous with one or more active regions of a neuroactive domain of ependymin are preferably homologous with active regions derived from the gamma-chain of ependymin. These preferred fragments, can be synthesized using an appropriate solid state synthetic procedure. J.M. Steward and F.D. Young, Solid Phase Peptide Synthesis, Freemantle, San Francisco, CA (1968). A preferred method is the Merrifield process. Merrifield, Recent Progress in Hormone Res. 23:451 (1967).

Further methods of preparing the fragments of this invention include well-known recombinant DNA methods. These methods comprise synthesizing the nucleic acids encoding the fragments of the invention, incorporating such nucleic acids via suitable cloning vectors into host cells and expressing the resulting polypeptides. T. Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982.

SUBSTITUTE SHEET

-31-

It will be appreciated by those skilled in the art that methods of preparing the protein fragments of this invention would also include preparing enzyme digests of other neuronotrophic proteins besides ependymin. Sequence analysis of proteolytic digests of these other neuronotrophic proteins and sequencing thereof, can be performed and the sequences derived from these neuronotrophic proteins can be compared with known ependymin sequences using computer database analysis.

This invention will now be illustrated by the following examples.

Example 1. Isolation and sequencing of gamma-ependymin and gamma-ependymin peptide fragments

Ependymin was isolated from goldfish brain as previously described. V.E. Shashoua, Neurochem. Res. 13: 649-655 (1988). Briefly, this method comprises isolating an ependymin-rich protein fraction of brain extracellular fluid by extracting brain tissue for 30 minutes at 0°C in a buffer consisting of sodium chloride, calcium acetate in Tris (pH 7.4) and L-1-tosylamide-2-phenylethylchloromethyl ketone as a protease and esterase inhibitor, V.E. Shashoua, Neurochem. Res. 6: 1129-1147 (1981). The gamma form of the protein was obtained by treating preparations of alpha and beta protein with peptide N-glyconase F

SUBSTITUTE SHEET

-32-

as previously described. Shashoua, V.E. Brain Res. 122: 113-124 (1977). The polypeptide chains, after reduction by heating for 1 min. at 95 C in the presence of 1% beta-mercaptoethanol and 1% sodium dodecyl sulfate (SDS) in Laemmli buffer, were separated by SDS gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes (Millipore Co., Bedford MA). After localization by Coomassie Blue staining, the bands were cut out and analysed for their N-terminal amino acid sequences using the Edman degradation and gas phase methods. Matsudaira, P., J. Biol. Chem., 262: 10035-10038.

The gamma-polypeptide chain was also electroblotted onto nitrocellulose membranes. These were cut out and treated with trypsin, chymotrypsin, or S. aureus protease to generate peptide fragments. Aebersold, R.H. et al., Proc. Natl. Acad. Sci, USA 84: 6970-6974 (1987). Each digest was separated by high pressure liquid chromatography into separate peptide fragments and detailed sequence analysis was performed using an Applied Biosystems model 470 sequencer (Microchemistry Laboratory, Dep't. Biology; Harvard College, Cambridge, MA.). The amino acid sequence data for 14 peptide fragments of gamma endodymin were derived in this way. All of these 14 peptide fragments correspond to identical sequences

-33-

that were previously found for the ependymin molecule by Konigstorfer et al., J. Neurochem. 52:310-312 (1989). The gamma-chain sequence obtained by sequence analysis of the peptide digests of the gamma ependymin chain is identical to that reported for the beta form of the molecule reported by Konigstorfer et al., supra.

Searches for homologies between the ependymin peptide fragments produced by these methods and known polypeptides were carried out using the Protein Identification Resource of the National Biomedical Research Foundation (Roode, D. et al. Nucl. Acids Res. 16: 1857-1859 (1988)) and the Swiss-Prot database at the University of Geneva.

Example 2. Effect of ependymin and ependymin fragments on nerve growth.

Tissue Culture Assay System

A tissue culture system has been developed to serve as a bioassay for ependymin fragments. The assay is based on the fact that certain cholinergic and adrenergic cell lines have the capacity to grow neurites and establish connections resembling synapses in vitro. Normally, such cells require about 4-7 days of culture before 50-80% of them begin to show neurite growth. This process can be accelerated by addition of drugs such as cyclic

SUBSTITUTE SHEET

-34-

adenosine monophosphate (cyclic AMP) to the culture medium.

Two cell lines were used. The mouse neuroblastoma cell line NB2a/d1 was grown to confluency in P/100 plastic plates in the presence of Delbecco's medium with 10% fetal calf serum at 36.5°C in a 5% CO₂ atmosphere. Shea, T.B. et al., Dev. Brain Res., 21: 307-314 (1985). An adrenergic mouse cell line based on PC12 pheochromocytoma cells was grown on collagen-coated culture plates using 85% RPMI 1640 and 10% heat-inactivated horse serum as the growth medium. Greene, L.A. et al., Proc. Natl. Acad. Sci. USA, 73: 2424-2428 (1976). Cultures were grown in P-100 plates from an initial plating of 10⁵ cells/ml under sterile conditions using 10 ml of incubation medium. Experimental cultures received a given concentration of protein fragment in 1ml of serum-free medium. Control cultures received no fragment. Equal numbers of test and control plates were incubated at 35.5°C in a 95%/5% air/CO₂ humidified atmosphere, and then examined for neurite growth patterns at 24 hr after the plating in a phase microscope at a magnification of 200 X.

The test to determine the degree of sprouting was carried out blind to determine (1) the total number of cells present in a given field of

SUBSTITUTE SHEET

-35-

observation and (2) the percent of these that have zero and one or two or more process. Twelve microscope fields, each containing 50-100 cells, were randomly selected in each plate and cells counted according to the categories described above. The data for 12 fields in each of two plates for each added protein fragment were combined to obtain an analysis of the percent sprouting (i.e. the % of cells that have one or more processes after 24 hr in culture) for each protein fragment. The ratio, % sprouting in experimental cultures/% sprouting in control cultures, is an index of the "relative sprouting effect" (RSE) of a protein fragment. In the counting, a cell had a single, distinct process if that process had a minimum length equal to at least 0.5 times the cell body length.

In each of the twelve microscope fields, the length of nerve cell processes (minimum length: 0.5 times the cell body length) was also measured to determine the percentage of nerve elongation after 24 hr. The percentage of total cells that developed a longest process equal to its cell body length was calculated according to the formula in Table V, supra. The test protein fragments derived from gamma-ependymin and their sequences are sequence I.D. numbers 8, 15, 16, 17, and 18, supra. The

SUBSTITUTE SHEET

-36-

following additives were also tested: pure ependymin, NGF and control protein fragment.

The gamma-chain fragments, some of which were initially isolated as the fragments from a trypsin digest of the gamma-chain of ependymin, as well as control fragments, were synthesized by the Merrifield process at the Peptide Synthesis Facility at Massachusetts General Hospital (Dr. G. Matsueda, Director). All the test additives were dissolved in phosphate buffered saline, filter sterilized and stored as 1 mg/ml stock solutions.

The data were reproducible, irrespective of the number of cells present in a given field of observation (Table IV).

-37-

TABLE IV
Neurite Sprouting Assay Data
 After 24 hrs in Culture

<u>Additive</u>	<u>NB2a/d1 cells</u>		<u>PC12 cells</u>	
	<u>Conc.</u> (μ M)	<u>RSE^a</u>	<u>Conc.</u> (μ M)	<u>RSE^a</u>
None		1.0	-	1.0
Ependymin (Dimer-from goldfish brain)	0.03	1.76 \pm .04		
	0.11	2.0 \pm .03	0.11	2.2
	0.60	2.0 \pm .03		
Nerve growth factor (7S-from mouse submaxillary gland)	0.03	1.76 \pm .034	-	-
	.07	1.76 \pm .07	.07	2.9
I.D. No. 27	0.1	1.3 \pm .05	1.0	1.58
	1	1.6 \pm .04		
	4.8	2.0 \pm .04		
I.D. No. 8	1.0	1.11 \pm .04	0.9	1.7
I.D. No. 15	1.0	1.1 \pm .04	1.0	2.6
I.D. No. 16			0.9	2.48
I.D. No. 17	1.0	1.39 \pm .05		
I.D. No. 18	0.5	1.5 \pm .03		
Control peptide ^c	1.0	1.03	0.9	1.07
dibutyryl cyclic AMP	1000	1.7	-	-

a. RSE = relative sprouting effect
 = S with additive / S without additive

S = % of cells that have one or more processes after 24 hrs in culture

A process = a cell extension equal to 0.1 cell body length

SUBSTITUTE SHEET

-38-

In experiments with two cell lines (Table IV), whole ependymin can promote the sprouting of neurites in the neuroblastoma cell line (NB2a/d1) and in the pheochromocytoma cell line (PC12).

Whole ependymin has comparable properties to nerve growth factor (NGF) in terms of initiating neurite sprouting. Specific fragments of the gamma-chain of ependymin have similar properties. Fragments with sequence I.D. Nos 15 and 16 both correspond to amino acid residues of the gamma ependymin chain that are substantially homologous to a portion of nerve growth factor. Measurements in the pheochromocytoma cell cultures show that both fragments could promote neurite sprouting. The concentrations for inducing neurite sprouting appear to be 10-15 fold higher than those required for intact nerve growth factor and intact ependymin. It is worth noting that both fragments were effective on the PC12 cells but had no significant effect on the neuroblastoma cells. Fragment with sequence I.D. No. 27 is associated with the nerve-growth promoting region of gamma-ependymin. This fragment, discussed in more detail below, was as effective as whole ependymin in promoting neurite sprouting. Other fragments such as sequence I.D. Nos. 8, 17, and 18 are fragments of the gamma-chain of ependymin that are associated with the matrix-promoting

SUBSTITUTE SHEET

-39-

neuroactive domains of the ependymin molecule (see Table I). These fragments were less effective in promoting neurite sprouting than the fragments from the nerve growth neuroactive domain (see Table II).

In an analogous manner, whole nerve growth factor had the most significant effects on neurite elongation (Table V). Fragments substantially homologous to the nerve growth neuroactive domain were similarly less effective in the neuroblastoma cell culture than in the PC12 cell culture, in terms of their effect on neurite elongation.

-40-

TABLE V
Neurite Elongation Assay Data
 After 24 hrs in culture

<u>Additive</u>	<u>NB2a/d1 cells</u>		<u>PC12 cells</u>	
	<u>Conc.</u> (μ M)	<u>REE^a</u>	<u>Conc.</u> (μ M)	<u>REE^a</u>
None		1.0	-	1.0
Ependymin	-	-	.03	1.74
Nerve Growth Factor	-	-	.03	3.30
I.D. No. 27	-	-	0.9	1.45
I.D. No. 8	0.9	1.47	0.9	1.72
I.D. No. 15	-	-	0.9	2.64
I.D. No. 16	-	-	0.2	2.18
I.D. No. 17	0.7	1.48	0.2	2.18
I.D. No. 18	0.5	1.80	0.5	1.55
dibutyryl cAMP	1000	2.58		

a. REE = relative elongation effect

$$= \frac{E \text{ with additive}}{E \text{ without additive}}$$

$$E = \sum x_1 l_1 + x_2 l_2 + x_3 l_3 \dots + x_n l_n$$

where:

x_1 = % of cells that have a process = one body length (l_1)

x_2 = % of cells that have a process = two body length (l_2)

x_n = % of cells that develop a longest process equal to n cell body lengths

SUBSTITUTE SHEET

-41-

Example 3. Dose-response Effects of ependymin and ependymin fragments (sequence I.D. Nos. 26, 27) on nerve growth.

This example illustrates the dose-response relationship of fragment I.D. No. 27 corresponding to a portion of the gamma-chain of ependymin in promoting nerve growth.

Tissue Culture Assay System

The tissue culture system described previously in Example 2 was used as a bioassay for ependymin and polypeptide fragment I.D. sequence number 27. Cells are dispersed in the assay medium and plated at a concentration of 10^5 cells/ml under sterile conditions into P-35 (6-well) dishes containing 2 ml of medium with or without a test additive. Equal numbers of test and control plates were incubated at 35.5°C in a 95%/5% air/CO₂ humidified atmosphere, and then examined for neurite growth at 18, 24 and 48 hr after the plating in a phase microscope at a magnification of 100 X. The test was carried out blind to determine (1) the total number of cells present in a given field of observation, (2) the percent of these that have one process and (3) the percent that have two or more processes. These three categories were determined in 5-7 randomly selected fields for each of two plates to obtain

-42-

mean and standard deviation data for each group. The results for an average of 4 experimental plates (containing an additive) were compared to those for 4 controls grown from the same stock culture; 3 experimental and 3 controls, each of which were grown on the same 6-well plate. The pattern of neurite outgrowth was determined as a function of additive concentration and time. The data were used to calculate the percent of cells remaining undifferentiated, i.e., those with zero processes (0), the percent with one process (1+), and the percent with two or more neurite processes (2+).

Sequence I.D. No. 26 initially isolated as a fragment from a trypsin digest of the gamma-chain of ependymin, was synthesized by the Merrifield process. Control peptide was synthesized, with the following sequence:

Lys Ala Ser Thr Thr Thr Asn Tyr Thr

All the test additives were dissolved in phosphate buffered saline, filter sterilized and stored as 1 mg/ml stock solutions.

The data were reproducible, irrespective of the number of cells present in a given field of observation. Statistical analysis using t-test comparisons of experimental vs. control cultures shows highly significant differences (p values of < 0.0005).

-43-

Ependymin alone causes a rapid growth of neurites. Approximately 70% of the cells of untreated control cultures remain undifferentiated (round cells) at the end of 48 hr in culture. Identical cultures treated with 12.5 $\mu\text{g/ml}$ ($\sim 2 \times 10^{-7}\text{M}$) ependymin had only about 20% undifferentiated cells after 48 hr in culture. The one-process category of cells (1+) was the same for both control and the ependymin-treated cultures. However about 80% of treated cells with ependymin had two or more nerve processes (2+), a 4-fold increase over control. The data are statistically significant in a t-test analysis with a p value of less than 0.0005. This demonstrates that native protein ependymin can significantly promote neurite growth.

Figure 1 shows the dose-response data for effects of ependymin on the pattern of development of cells with two or more processes (2+) after 24 hr in culture. The data show that the half-maximal effective dose occurs at a concentration of about 2 $\mu\text{g/ml}$ or $30 \times 10^{-9}\text{M}$. Also, there seems to be a plateau above a dose of 7.5 $\mu\text{g/ml}$, suggesting that there is a saturation of the response to the effects of ependymin. Thus, increased amounts beyond this level appear to cause no further enhancement of neurite growth.

SUBSTITUTE SHEET

-44-

Fragment sequence I.D. No. 26 was isolated from tryptic digests of ependymin and corresponds to a fragment of the molecule present at positions 80 to 85 in the sequence of the gamma-chain of ependymin. It was synthesized as sequence I.D. No. 27 and used in assays, testing its capacity to potentiate growth of the neuroblastoma NB2a/d1 cells in culture. The peptide has a molecular weight of 1034 and is highly soluble in saline at physiological pH values.

The polypeptide fragment of I.D. No. 27 was highly effective in promoting neurite outgrowth. The effects of a dose of 2.5 µg/ml (2.4×10^{-9} M) revealed a reproducible and pronounced decrease in the number of undifferentiated cells (0 processes) and an increase in the number of cells with more than two processes (2+) with no change in the one-process category. The data for (0) and (2+) were statistically significant in a t-test comparison (p value of 0.0005) with those for untreated control cells. The effect of the polypeptide was readily observable after only 1-2 days in culture. The time course of development of type (2+) cells for a dose of 1.5 µg/ml of the peptide showed that within 24 hr, 60-80% of the cells plated developed neurites as compared to 20-30% in control plates. The results were highly reproducible. T-test comparisons vs. controls gave

SUBSTITUTE SHEET

-45-

p values of 0.0005.

Figure 2 shows dose-response data for this polypeptide. There is an initial step function with a half-maximal effect of 0.004 $\mu\text{g/ml}$ ($3.8 \times 10^{-9}\text{M}$) followed by a gradual linear increase as a function of concentration. If we calculate the ependymin equivalent by multiplying the half-maximal dose by the molecular weight ratios of native ependymin to sequence I.D. No. 27 (i.e., 68,000/1034) we find that the corresponding ependymin dose would be 0.263 $\mu\text{g/ml}$. This is a factor of about 10 less than the half-maximal dose for stimulating cell growth by pure ependymin (Figure 1). Thus fragment No. 27 appears to be 10-fold more active than ependymin itself.

Pretreatment of cells for a short period of time can induce a neurite outgrowth effect which persists after this polypeptide is removed from the culture. A series of experiments were performed in which cells were treated with 0.1 $\mu\text{g/ml}$ of fragment I.D. No. 27 for 3 or 18 hr. The medium was then removed and replaced with control medium and the growth patterns of the cultures were examined after 24 and 48 hr in culture. The results showed that exposure for a 3-hr pulse of this polypeptide results in an enhanced growth which persists for 48 hr in

SUBSTITUTE SHEET

-46-

comparison with controls. This suggests that this polypeptide might function as an initiation signal for neurite development.

The effect of this polypeptide does not appear to be due to an inhibition of protease activity. It is well established that inhibitors of proteases can promote the growth of neurites in culture. D. Monard, Biochem. Pharm. 36: 1389-1392 (1987). A comparison of the effect of this polypeptide at a dose of 1 µg/ml with a similar concentration of control was performed. In these experiments, the cultures were plated without any trypsin treatment to avoid effects that may arise from any residual proteases in the culture. The cells were washed in phosphate-buffered saline and grown for 24 hr. The results showed a clear increase in the (2+) type of cells with added fragment I.D. No. 27 whereas the data for those treated with a control peptide are not significantly different from those of untreated controls.

Example 4: Effect of Secondary molecule-Linked Ependymin Fragments on Nerve Growth

This Example illustrates that a prodrug comprising the protein fragment I.D. 27 linked to a secondary molecule is capable of promoting nerve growth with no toxic effects.

-47-

A. Synthesis of DHA Derivatives of Sequence No. 27

1. Synthesis of docosohexa-enoic anhydride (DHA anhydride)

One gram of docosohexa-enoic acid DHA (0.0003 M) was dissolved in 100ml of methylene chloride and 50 ml benzene. The mixture was stirred together with 0.36 g dicyclohexyl carbodiimide (DCC) for about 16 hr at room temperature. A white precipitate of dicyclohexyl urea formed. This was filtered off and the solution of the anhydride was then concentrated to a volume of 20 ml and refiltered to remove additional dicyclohexylurea. Aliquots of this solution were then used directly in the next stage of the synthesis.

2. Coupling of DHA anhydride with protein fragment I.D. 26

Ten milligrams of protein fragment I.D. No. 27 (MW 1034, 9.6×10^{-6} M) was dissolved in 10 ml of N-methylpyrrolidone containing 4.7 mg of N-dimethylamino pyridine (DMAP). A solution of the DHA anhydride (9.6×10^{-6} M) in 0.14 ml benzene was then added and the mixture was stirred at room temperature for about 48 hrs. The pale yellow solution was filtered and evaporated to dryness at 37°C at reduced pressure (0.1 mm Hg). The product

SUBSTITUTE SHEET

-48-

was then extracted 3x with 10 ml aliquots of hexane and then evaporated to dryness. The residue was dissolved in 5 ml of 1M acetic acid and extracted 3x with 10 ml aliquots of n-hexane followed by 3 additional extractions with 10 ml benzene to remove the DHA liberated in the reaction. The remaining solution was evaporated to dryness in a rotary evaporator to give a pale yellow viscous oil.

The product migrated as a single spot on a thin layer chromatogram as a compound with an Rf of 0.52 (fragment I.D. No. 27: Rf 0.25) using chloroform/methanol/acetic acid (77.5/15/7.5) as the elution solvent. The IR spectrum of the compound was consistent with the proposed mono-DHA derivative of the polypeptide. Strong absorption bands were found at: 3375, 3050, 1650, 1440, 1280, 1070, and 980 cm. The compound was water soluble.

A similar procedure was used to prepare the di-substituted DHA derivative of fragment I.D. No. 27. This had an Rf value of 0.9 on thin layer chromatograms using chloroform/methanol/acetic acid (77.5/15/7.5) as the solvent. Its IR spectrum was consistent with a di-substituted peptide. It was soluble in 70/30 ethanol/water.

B. Results

The tissue culture assay system used in this

-49-

Example is identical to that described previously in Example 3.

The mono, and di-docosahexa-enoic acid derivatives of fragment I.D. No. 27 were prepared as described above.

The protein fragment alone was highly effective in promoting neurite outgrowth. In a manner identical to that of Example 3, administration of this ependymin fragment produced a marked decrease in the number of undifferentiated cells (0 processes) and an increase in the number of cells of more than two processes (2+) (Figure 3A). The effect of the mono-acid derivative of this polypeptide was even more pronounced. (Figure 3B). The effect of the di-docosahexa-enoic acid derivative on neurite outgrowth was not significantly different from that of the polypeptide alone. (Figure 3C).

This Example illustrates that secondary molecules capable of crossing the blood-brain barrier can be coupled with protein fragments of this invention to yield prodrugs that are non-toxic. Moreover, such prodrugs can show greater nerve-growth promoting activity than the fragment alone.

The molecules of this invention including ependymin fragments that are substantially

SUBSTITUTE SHEET

-50-

homologous to an active region of a neuroactive ependymin domain, may be useful as treatments to enhance recovery after stroke, as a factor that promotes neurite outgrowth and regeneration. These molecules might be useful in enhancing long term memory capacity that is lost during the normal process of aging. Thus, molecules of this invention may restore the aging-related decline in capacity of brain cells to form new connections.

Molecules of this invention can also be useful in treatment in brain degenerative processes to promote the formation of neuronal connections broken during the onset and development of Huntington's, Parkinson's and Alzheimer's diseases. Molecules of this invention may also be useful in studies of transplantation of neurons. Pre-treatment of neurons with these protein fragments or transplantation in the presence of the protein fragments may enhance connectivity and growth.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentations, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed in the scope of the following claims.

-51-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

Shashoua, Victor E.

(ii) TITLE OF INVENTION:

Nerve Growth Peptides and Uses Therefor

(iii) NUMBER OF SEQUENCES:

27

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MA

SUBSTITUTE SHEET

-52-

(E) COUNTRY:
United States of America

(F) ZIP:
02146

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:
Diskette, 5.25 inch

(B) COMPUTER:
IBM-compatible

(C) OPERATING SYSTEM:
MS-DOS Version 3.3

(D) SOFTWARE:
WordPerfect

SUBSTITUTE SHEET

-53-

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
6 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1
Asp Leu Gln Val Phe Val
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
5 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2
Gly Glu Lys Lys Asp
1 5

SUBSTITUTE SHEET

-54-

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
5 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3
Ala Thr His Glu Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
6 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4
Cys Leu Pro ____ Val Ser Gly
1 5

-55-

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
6 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5
Leu Pro Val Ser Gly Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
6 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6
Pro Cys His Ala Pro Pro
1 5

SUBSTITUTE SHEET

-56-

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
8 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7
Gly Gly His Asp Leu Glu Ser Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
4 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8
Asp Asp Leu Gln — Val Phe
1 5

SUBSTITUTE SHEET

-57-

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
5 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

Pro Leu Thr ____ Ser Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
8 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

Leu Ile His Phe Glu Glu Gly Val
1 5

-58-

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
7 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11
Gly Glu Phe Ser Tyr Asp Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
7 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12
His Ala Pro Pro Leu Thr Ser
1 5

-59-

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
7 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13
Asp Leu Glu Ser Gly Glu Phe
1 5

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
8 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14
Gly Glu Phe Ser Val Cys Asp Ser
1 5

SUBSTITUTE SHEET

-60-

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
10 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15
Lys Lys Gly Glu Phe Ser Val Ala Asp Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
9 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16
Lys Lys Gly Glu Phe Tyr Cys Ser Arg
1 5

SUBSTITUTE SHEET

-61-

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
13 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY:
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17

Gly Leu Arg Val Arg Val Trp Asn Gly Lys Phe Pro Lys
 1 5 10

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
16 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY:
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18

Gly Val Ala Phe Glu Glu Ala Pro Asp Asp His Ser Phe
 1 5 10
 Phe Leu Phe
 15

-62-

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
7 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19
Gly Gly His Asp Leu Ser Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
7 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20
Gly Gly His Asp Leu Glu Ser Gly
1 5

-63-

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:
14 amino acids

(B) TYPE:
amino acid

(D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21

1 Gly Gly His Asp Leu Glu Ser Gly Glu Phe Ser Tyr Asp Ser

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:
14 amino acids

(B) TYPE:
amino acid

(D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22

Gly Gly Ser Asp Leu Ser Gly Glu Phe Ser Val Cys Asp Ser
1 5 10

-64-

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
15 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23

Gly Gly Ser Asp Leu Ser Gly Gly Glu Phe Ser Val Cys
 1 5 10
 Asp Ser
 15

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
15 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24

Gly Gly Ser Asp Leu Ser Gly Gly Glu Phe Ser Val Ala
 1 5 10
 Asp Ser
 15

-65-

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
14 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25

Gly	Gly	Ser	Asp	Leu	Ser	Gly	Glu	Phe	Ser	Val	Ala
1				5					10		
Asp	Ser										

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
6 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26

Glu	Thr	Leu	Gln	Phe	Arg
1			5		

SUBSTITUTE SHEET

-66-

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:
8 amino acids
- (B) TYPE:
amino acid
- (D) TOPOLOGY
linear

(ii) MOLECULE TYPE:
peptide(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27
Lys Lys Glu Thr Leu Gln Phe Arg
1 5

-67-

CLAIMS

1. A protein fragment capable of stimulating nerve growth, the fragment including a sequence substantially homologous with an active region of at least one neuroactive domain of ependymin.

2. The fragment of claim 1, wherein the sequence is between about 5 and about 16 amino acids in length.

3. The fragment of claim 2, wherein the sequence is between about 5 and about 8 amino acids in length.

4. The fragment of claim 1, wherein the sequence of the protein fragment is at least about 70% homologous with the active region of the neuroactive domain of ependymin.

5. The fragment of claim 4, wherein the protein fragment is a portion of a neuronotrophic protein, the neuronotrophic protein selected from the group consisting of ependymin, mouse epidermal growth factor, human epidermal growth factor, human epidermal growth factor receptor, human epidermal growth factor precursor, nerve growth factor, nerve

-68-

growth factor precursor, human laminin B1, mouse laminin B1, tubulin, fibronectin, and integrin.

6. The fragment of claim 1, wherein the neuroactive domain of ependymin comprises amino acid sequences selected from the group consisting of amino acids 1 through about 80 and amino acids 130 through about 195.

7. The fragment of claims 1 or 6, wherein the active region comprises amino acids selected from the group consisting of amino acid sequence I.D. numbers 1 to 13 and 26.

8. The fragment of claim 7, further comprising a secondary molecule coupled to the protein fragment.

9. The fragment of claim 8, further comprising at least one basic amino acid linked to the fragment by at least one hydrolyzable bond.

10. The fragment of claim 9, wherein the secondary molecule can cross the blood-brain barrier.

11. The fragment of claim 10, comprising an amino acid sequence selected from the group consisting of sequence ID numbers 1 to 27.

-69-

12. The fragment of claim 11, further comprising a secondary molecule coupled to the protein fragment.

13. A protein fragment capable of stimulating nerve growth selected from the group consisting of amino acid sequence ID numbers 1 through 27.

14. An ependymin fragment capable of stimulating nerve growth, the fragment comprising at least 5 amino acids from a neuroactive domain of ependymin, said fragment substantially homologous to a portion of at least one neuronotrophic protein other than ependymin.

15. The fragment of claim 14, further comprising a secondary molecule coupled to the ependymin fragment.

16. The fragment of claim 15, wherein the secondary molecule is linked to the fragment by at least one basic amino acid, said linkage including at least one hydrolysable bond.

17. The fragment of claim 15, further comprising at least one basic amino acid linked to the fragment by at least one hydrolysable bond.

SUBSTITUTE SHEET

-70-

18. The fragment of claim 16, wherein the secondary molecule is a pharmaceutically acceptable carrier.

19. The fragment of claim 17, further comprising a pharmaceutically acceptable carrier that is coupled to least one of said basic amino acids.

20. The fragment of claims 18 or 19, wherein the pharmaceutically acceptable carrier is capable of crossing the blood-brain barrier.

21. The fragment of claim 14, wherein the fragment comprises amino acid sequences selected from the group consisting of sequence I.D. numbers 1 to 27.

22. The fragment of claim 20, wherein the fragment comprises amino acid sequences selected from the group consisting of sequence I.D. numbers 1 to 27.

23. A method of treating a patient having a nerve injury, comprising administering to the patient at least one protein fragment capable of stimulating nerve growth, the fragment having a

-71-

sequence substantially homologous with an active region of a neuroactive domain of ependymin.

24. The method of claim 23, wherein the step of administering includes administering a protein fragment between about 5 and about 16 amino acids in length.

25. The method of claim 23, wherein the step of administering includes administering at least one protein fragment of a neuronotrophic protein selected from the group consisting of ependymin, mouse epidermal growth factor, human epidermal growth factor, human epidermal growth factor receptor, human epidermal growth factor precursor, nerve growth factor, nerve growth factor precursor, human laminin B1 mouse laminin B1, tubulin, fibronectin, and integrin.

26. The method of claim 23, wherein the neuroactive domain of ependymin comprises amino acid sequences selected from the group consisting of amino acids 1 through about 80 and amino acids 140 through about 180.

27. The method of claim 26, wherein the active region of ependymin comprises amino acid sequences

SUBSTITUTE SHEET

-72-

selected from the group selected from sequence I.D. numbers 1 to 13 and 26.

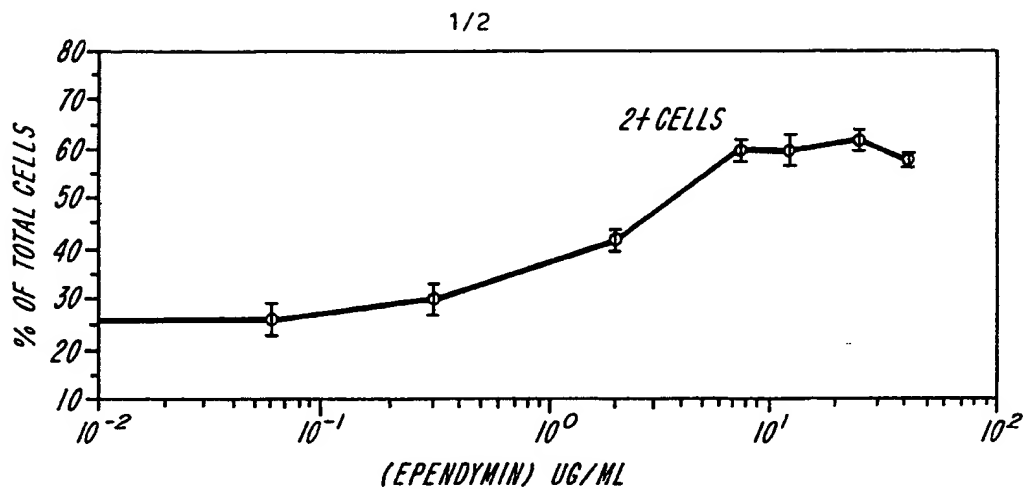
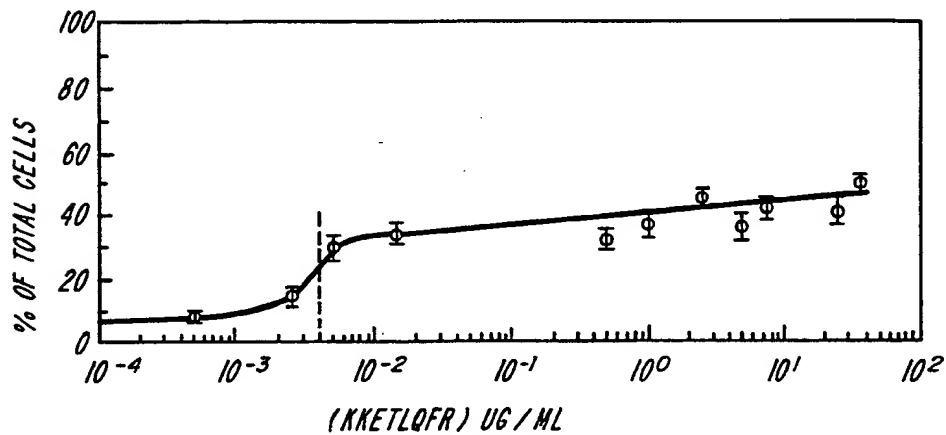
28. The method of claims 24 or 27, further comprising the step of coupling the protein fragment to a secondary molecule prior to administration.

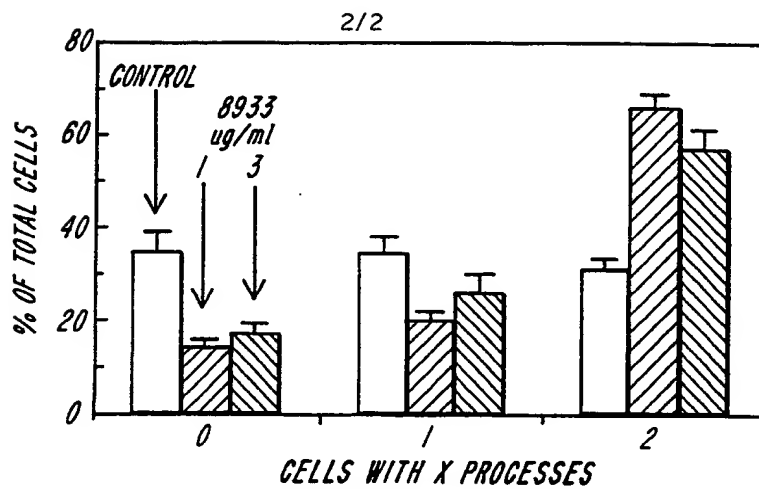
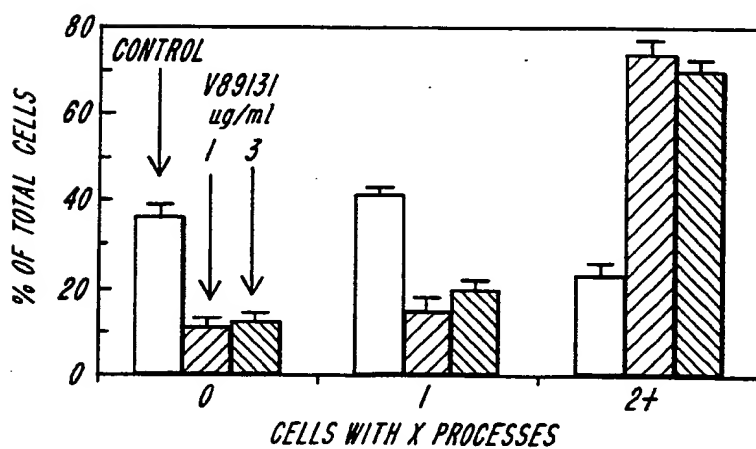
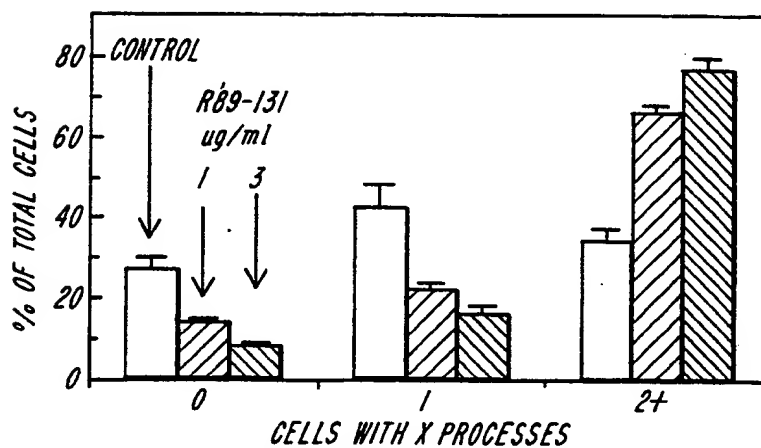
29. The method of claim 28, wherein the secondary molecule is a pharmaceutically acceptable carrier that can cross the blood-brain barrier.

30. The method of claim 23, wherein the step of administering the protein fragment comprises providing to the patient a sustained-release device containing the protein fragment.

31. The method of claim 24, wherein the protein fragment is selected from the group consisting of sequence I.D. numbers 1 to 27.

32. The fragment of claim 19, wherein said pharmaceutically acceptable carrier is coupled at an N-terminal end and at a C-terminal end of the fragment by way of said basic amino acids.

**FIG. 1****FIG. 2**

**FIG. 3A****FIG. 3B****FIG. 3C**
SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US91/03346

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 37/02; C07K 7/06 U.S.Cl.: 514/15; 530/329											
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ?</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="height: 40px; vertical-align: bottom;">U.S.</td> <td style="vertical-align: bottom;">514/15; 530/329</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *</div> <div style="padding: 5px;">APS, CAS, Biosis</div>			Classification System	Classification Symbols	U.S.	514/15; 530/329					
Classification System	Classification Symbols										
U.S.	514/15; 530/329										
III. DOCUMENTS CONSIDERED TO BE RELEVANT * <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category *</th> <th style="width: 60%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">WO/A, 89/07938 (SHASHOUA) 08 September 1989. See entire document.</td> <td style="vertical-align: top;">8-11, 15-20, 32</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">The EMBO Journal, vol. 6. No. 8, issued 1987, Rocco et al., "Models of Fibronectin" pages 2343-2349, see page 2346.</td> <td style="vertical-align: top;">1-13</td> </tr> </tbody> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	WO/A, 89/07938 (SHASHOUA) 08 September 1989. See entire document.	8-11, 15-20, 32	Y	The EMBO Journal, vol. 6. No. 8, issued 1987, Rocco et al., "Models of Fibronectin" pages 2343-2349, see page 2346.	1-13
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Y	The EMBO Journal, vol. 6. No. 8, issued 1987, Rocco et al., "Models of Fibronectin" pages 2343-2349, see page 2346.	1-13									
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>											
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="text-align: center; padding-top: 10px;">08 August 1991</td> <td style="text-align: center; padding-top: 10px;">06 SEP 1991</td> </tr> <tr> <td style="border-bottom: 1px solid black;">International Searching Authority</td> <td style="border-bottom: 1px solid black;">Signature of Authorized Officer</td> </tr> <tr> <td style="text-align: center; padding-top: 10px;">ISA/US</td> <td style="text-align: center; padding-top: 10px;"> S. G. Marshall </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	08 August 1991	06 SEP 1991	International Searching Authority	Signature of Authorized Officer	ISA/US	 S. G. Marshall	
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08 August 1991	06 SEP 1991										
International Searching Authority	Signature of Authorized Officer										
ISA/US	 S. G. Marshall										

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category ^a	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Journal of Biological Chemistry, vol. 264, No. 23, issued 15 August 1989, Konigstorter et al., "Biosynthesis of Ependymins from Gold fish Brain," pages 13689-13692, see pages 13690 and 13692.	14-22.32
Y	US, A, 4,868,161 (Roberts) 19 September 1989, see entire document.	23-31